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Dietary cholesterol-oxidation products accumulate in serum and liver in apolipoprotein E-deficient mice, but do not accelerate atherosclerosis

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There are conflicting reports regarding the effect of dietary cholesterol-oxidation products (oxysterols) on the development of atherosclerosis in experimental animals. To address this issue, apolipoprotein (Apo) E-deficient mice were fed a purified diet (AIN-93) or the same purified diet containing 0·2 g cholesterol or 0·2 g oxysterols/kg. The dietary oxysterols had no significant effect on the serum lipid levels. Although all of the diet-derived oxysterols (cholest-5-en-3β,7α-diol, cholest-5-en-3β,7β-diol, cholestan-5α,6α-epoxy-3β-ol, cholestan-5β,6β-epoxy-3β-ol, cholestan-3β, 5α, 6β-triol, cholest-5-en-3β-ol-7-one and cholest-5-en-3β, 25-diol) accumulated in the serum and liver, only cholest-5-en-3β-ol-7-one and cholestan-3β, 5α, 6β-triol accumulated significantly ($P<0·05$) in the aorta. The oxysterol diet did not result in elevation of the aortic cholesterol level or the lesion volume in the aortic valve. These present results indicate that exogenous oxysterols do not promote the development of atherosclerosis in ApoE-deficient mice.

There are conflicting reports as to whether diet-derived cholesterol-oxidation products (oxysterols) initiate early atherosclerotic lesion and accelerate the lesion to the advanced lesion, as reviewed by Brown & Jessup (1999) and Schroepfer (2000). According to Brown & Jessup (1999), of thirteen studies using rabbits, quail, pigeons, rats, cockerels, hares and chickens, six indicated a pro-atherogenic effect of dietary oxysterols (Cook & MacDougall, 1968; Imai *et al.* 1976; Shih, 1980; Jacobson *et al.* 1985; Matthias *et al.* 1987; Mahfouz *et al.* 1997), four an antiatherogenic effect (Aramaki *et al.* 1967; Griminger & Fisher, 1986; Higley *et al.* 1986; Tipton *et al.* 1987) and three indicated no clear-cut action (Kantiengar *et al.* 1955; Imai & Lee, 1983; Sunde & Lalich, 1985). According to Brown & Jessup (1999), the reasons for these discrepancies are not clear, but they cannot be explained by study design or duration, dosage or type of oxysterols fed, or the animal model employed.

More recently, Staprans *et al.* (2000) demonstrated that oxysterols in a diet accelerated fatty streak lesion formation in the aorta of both LDL receptor- and apolipoprotein (Apo) E-deficient mice, which developed extensive atherosclerosis. The dietary oxysterols increased in the sera of these mutant mice, but precise information on the composition and tissue levels of these oxysterols was not reported. In the present study, therefore, we fed ApoE-deficient mice a purified diet and the same purified diet containing cholesterol or oxysterols and measured the oxysterol levels in the aorta, serum and liver by GC–MS. The effect of these diets on the aortic cholesterol level and the lesion volume in the aortic valve was also measured.

Materials and methods

Materials

5α-Cholestane, cholest-5-en-3β-ol-7-one (7-ketocholesterol), cholestan-3β,5α,6β-triol (cholestanetriol), cholestan-5α,6α-epoxy-3β-ol (α-epoxycholesterol) and cholest-5-en-3β,25-diol (25-hydroxycholesterol) were purchased from Sigma Chemical Co., St Louis, MO, USA. Cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), cholestan-5β,6β-epoxy-3β-ol (β-epoxycholesterol), and 25-*R*-cholest-5-en-3β,26-diol (27-hydroxycholesterol) were obtained from Steraloids Inc., Wilton, NH, USA. Cholest-5-en-3β-ol (cholesterol) was

Abbreviations: Apo, apolipoprotein; cholestanetriol, cholestan-3β,5α,6β-triol; cholesterol, cholest-5-en-3β-ol; α-epoxycholesterol, cholestan-5α,6α-epoxy-3β-ol; β-epoxycholesterol, cholestan-5β,6β-epoxy-3β-ol; 7α-hydroxycholesterol, cholest-5-en-3β, 7α-diol; 7β-hydroxycholesterol, cholest-5-en-3β,7β-diol; 25-hydroxycholesterol, cholest-5-en-3β,25-diol; 27-hydroxycholesterol, 25-*R*-cholest-5-en-3β,26-diol; 7-ketocholesterol, cholest-5-en-3β-ol-7-one; oxysterols, cholesterol-oxidation products.

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purchased from Daiichikagaku, Tokyo, Japan. Pyridine, trimethylchlorosilane and 1,1,1,3,3,3-hexamethyldisilazane were the products of Nacalai Tesque, Kyoto, Japan. All organic solvents were of guaranteed reagent standard. Oxysterols were prepared by heating cholesterol at 150°C for 12 h (Osada *et al.* 1994). The heated products were applied on a silicic acid (Silica Gel 60, 70–230 mesh; Merck-Japan, Tokyo, Japan) column ($3\,cm^2 \times 30\,cm$) and fractionated by successive elution with 100 ml of *n*-hexane, 100 ml diethyl ether (50 ml/l hexane), and finally with 200 ml methanol (Dzeletovic *et al.* 1995). The polar fraction rich in oxysterols was eluted with methanol. This operation was repeated three times. The oxysterols were analysed by GC–MS using SCAN mode (Shimadzu, Kyoto, Japan). The oxysterols contained 8 g cholesterol/kg, and a large amount of unidentified polar compounds (205 g/kg) (Table 1). The most abundant oxysterol was 7-ketocholesterol, followed by α-epoxycholesterol, β-epoxycholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol, 25-hydroxycholesterol and then cholestanetriol.

Animals and diets

Each of the male and female ApoE-deficient mice initially engineered at the University of North Carolina (Chapel Hill, NC, USA) was purchased from Jackson Laboratory (Bar Harbor, ME, USA) in 1994 (Ni *et al.* 1998). These mice were maintained at the Laboratory of Animal Experiments in Kyushu University Faculty of Medicine (Fukuoka, Japan). ApoE-deficient mice (9-week-old) were housed individually in a temperature-controlled room at 22–25°C with a 12 h light–dark cycle (light on, 08.00–20.00 hours) and given free access to diet and deionized water throughout the experimental period.

Male ApoE-deficient mice (9–10-week-old) with an initial weight of 25·6 (SE 0·5) g were divided into three groups of five mice each according to the difference in diet, and the animals were given free access to purified diets for 8 weeks. These three groups of mice were fed either a purified diet (control diet), the purified diet containing 0·2 g cholesterol/kg (cholesterol diet) or the purified diet containing 0·2 g oxysterols/kg (oxysterol diet). The purified diet was prepared according to the formula recommended by the American Institute of Nutrition (Reeves *et al.* 1993) and contained (g/kg): casein 200·0, palm oil 100·0, α-maize starch 132·0, sucrose 100·0, cellulose 50·0, vitamin mixture 10·0, mineral mixture 35·0, L-cysteine 3·0, choline bitartrate 2·5, *tert*-butyl hydroquinone 0·014, and maize starch to 1000 g. Cholesterol or oxysterols were added at the expense of maize starch. Experimental diets were packed in a pouch containing an O_2 absorbent (Ageless S-200; Mitsubishi Gas Chemical Co., Tokyo, Japan), flushed with N_2 and stored at 4°C. The diet was freshly prepared every week, and changed every 2 d, with any remaining diet being discarded. At the end of the feeding period, the mice were deprived of food for 4 h prior to sacrifice.

Table 1. Composition of cholesterol-oxidation products (oxysterols) in the diet*

(Mean values for determinations in triplicate)

Oxysterol	g/kg total oxysterols
Cholesterol	8
7α-Hydroxycholesterol	56
7β-Hydroxycholesterol	152
β-Epoxycholesterol	156
α-Epoxycholesterol	162
Cholestanetriol	13
7-Ketocholesterol	221
25-Hydroxycholesterol	27
Unknown	205

* For details of analytical procedures, see p. 341.

Cholesterol, cholest-5-en-3β-01, 7α-hydroxycholesterol, cholest-5-en-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-en-3β,7β-diol; β-epoxycholesterol, cholestan-5β,6β-epoxy-3β-01; cholestanetriol, cholestan-3β,5α,6β-triol, 7-ketocholesterol, cholest-5-en-3β-01-7-one; 25-hydroxycholesterol, cholest-5-en-3β,25-diol.

Tissue preparation and morphometric determination of atherosclerosis

The animals were anaesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and killed by withdrawing blood from the left ventricle. The blood was transferred into 1 ml microcentrifuge tubes containing 50 μg butylated hydroxytoluene. The serum was collected after centrifugation (GEAVI DNA; Sanyodennki Co., Tokyo, Japan) at 2000 ***g*** for 20 min. N_2 was bubbled through the serum, which then was stored at −80°C before being analysed. The livers and aortas were removed and immediately placed in ice-cold PBS (1 ml/l) and stored at −80°C until analysis. The adipose tissue around the aorta was dissected away, rinsed in fresh PBS, blotted dry between filter paper, snap frozen in liquid N_2, and stored at −80°C.

The circulatory system was perfused with 50 ml PBS, pH 7·4, via a cannula inserted into the left ventricle, allowing unrestricted efflux from an incision in the vena cava. After the aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, perfusion fix for the heart was immediately continued with 50 ml neutral formalin buffer solution (100 ml/l), pH 7·4 The heart was removed and fixed in neutral formalin buffered solution (100 ml/l) (Ni *et al.* 1998). To determine the cross-sectional lesion volume, hearts containing aortic roots were processed for quantitative atherosclerosis assay of the aortic root using a modification of the methods described by Paigen *et al.* (1987). Briefly, the heart was cut along a plane between the tip of the two atria and the top half was embedded in paraffin. Consecutive sections (10 μm thick) were prepared from the ascending aorta to the aortic sinus until the aortic tissue disappeared. The sections were mounted on glass slides and stained with elastic Van Gieson and haematoxylin (Ni *et al.* 1998). Five sections of each heart were selected for intimal area determination; the first and most distal sections to the heart were taken where the aortic valve cusp was barely discernible. From this section, moving to the base of the heart, the 15th, 30th, 45th and 60th sections were also photographed together with the first one, using a video camera mounted on an Olympus LX70 light microscope (Tokyo, Japan) and analysed using Adobe Photoshop (San Joje, CA, USA) and NIH image/68 k 1·57 software (National

Institutes of Health, Bethesda, MD, USA) on a Power Macintosh computer. The mean intimal area was calculated for each animal and subsequently for each group.

Determination of oxysterols

To 200 μl serum and 0·25 g liver, 50 μg 5α-cholestane (100 μg/ml ethanol) as an internal standard was added using a transfer pipette. To total aorta, 5 μg 5α-cholestane (10 μg/ml ethanol) was added in the same way. Lipids were extracted with 20 vol. Chloroform–methanol (2:1, v/v) containing 0·1 ml butylated hydroxytoluene/l according to the method of Folch *et al.* (1957). The screw-capped tube was flushed with Ar (purity 99·9 %; Hakata Kyoudousannso Co., Fukuoka, Japan) and 4 ml freshly prepared 1 M-ethanolic KOH solution was added. The samples were allowed to saponify at room temperature overnight in the dark (Mori *et al.* 1996). To the saponified samples, 4 ml water was added and the unsaponified lipids were extracted three times with 4 ml hexane. A part of the residue was used for the determination of cholesterol (Nagao *et al.* 2001). The rest was dried under N_2 and the residue was dissolved in 1 ml toluene (guaranteed reagent; Nacalai Tesque, Kyoto, Japan).

Cholesterol was separated from the oxysterols using solid-phase extraction (Dzeletovic *et al.* 1995), since it interfered with the determination of oxysterols. Sep-Pak Vac silica cartridge (Waters Japan, Tokyo, Japan) was conditioned with 2 ml hexane. A sample in 1 ml toluene was applied to the silica cartridge using a Pasteur pipette. After a 1·5 ml hexane wash, the cholesterol was eluted with 8 ml 2-propanol (5 ml/l hexane). The cholesterol fraction was discarded. Oxysterols were then eluted with 5 ml 2-propanol (300 ml/l hexane). Since 5α-cholestane was eluted together with cholesterol, 5α-cholestane was added to the eluates from the serum (500 ng), liver (500 ng) and aorta (50 ng), as an internal standard.

After removal of the oxysterol fraction-containing solvent under N_2, the dried residues were converted to trimethylsilyl ethers *tert*-butyl hydroquinone–1,1,1,3,3,3-hexamethyldisilazane–dried pyridine (1:3:9, by vol.) for 30 min at room temperature. The samples were dried under N_2 and dissolved in hexane (50 μl) for GC–MS analysis.

GC–MS was performed on a Shimadzu GC-17A version 3 GC (Shimazu) coupled with an SPB-1 fused silica capillary column (60 m × 0·25 mm, 0·25 μm phase thickness; Supelco Inc., Bellefonte, PA, USA), connected to a Shimadzu QP5050A series mass-selective detector. The oven temperature program was as follows: 180°C for 1 min, 20°C/min at 250°C, and then 5°C/min at 290°C where the temperature was maintained for 30 min. The carrier gas was He. The total run time was 42·5 min. The GC was operated in the constant flow mode, with the flow rate set to 1·5 ml He/min. The injector was operated in the split ratio 1:5 and was kept at 300°C and the detector transfer line was kept at 250°C. The MS was operated in the electron impact mode (70 eV). Quantitative analysis was performed by the internal standard method for MS using a selected ion monitoring mode (Mori *et al.* 1996). The monitored ion during the chromatographic run was varied as a function of time and the characteristic ion for each oxysterol was recorded. To quantify the oxysterol calibration curves, measurement of the peak area of the oxysterol *v.* that of the internal standard was used. Peak identification was confirmed by relative retention time and mass spectral comparison with authentic standards, as well as with the NIST/EPA/NIH mass spectral database library (NIST'02 ASC1 version, Gaithersburg, MD, USA). As reported elsewhere (Tomoyori *et al.* 2002), oxysterols were grouped into seven 'time windows': group 1 17–20 min (5α-cholestane); group 2 20–27 min (7α-hydroxycholesterol); group 3 27–29·7 min (7β-hydroxycholesterol, β-epoxycholesterol, α-epoxycholesterol); group 4 29·7–32 min (unidentified compounds); group 5 32–34·2 min (cholestanetriol); group 6 34·2–36·5 min (7-ketocholesterol, 25-hydroxycholesterol); group 7 36·5–42·5 min (27-hydroxycholesterol). Several variables including ions monitored, relative retention time, correlation coefficient for calibration curves, response factors for the monitored ions, detection limit and CV for repeated injections have been reported elsewhere (Tomoyori *et al.* 2002).

Analyses of serum and liver lipids and α-tocopherol

The serum lipid levels were determined using commercially available kits (Cholesterol C test, Triglyceride G Test and Phospholipid B Test; Wako Pure Chemicals, Osaka, Japan) as previously described (Nagao *et al.* 2001). Liver cholesterol, triacylglcyerols and phospholipids were chemically determined as previously described (Nagao *et al.* 2001). Serum and liver α-tocopherol concentrations were determined by HPLC as previously described (Nagao *et al.* 2001).

Statistical analysis

Statistical analyses were carried out using Statcel (Excel 2000, Microsoft). Variance among the groups was first tested according to the Bartlett test. Statistical differences were analysed by the Fisher's PLSD method.

Results

The diets had no significant effect on the body weight gain, food intake and liver weight in ApoE-deficient mice (results not shown).

There were no significant differences in the levels of serum and liver lipids in the ApoE-deficient mice fed the control, cholesterol and oxysterol diets, except for a significantly ($P<0·05$) higher level of liver cholesterol in the ApoE-deficient mice fed the cholesterol diet (Table 2). Serum and liver α-tocopherol levels tended to be lower in the oxysterol group than in the control and cholesterol group, but the difference was not significant.

Table 3 shows the level of cholesterol and oxysterols in the aorta of ApoE-deficient mice fed the control, cholesterol and oxysterol diets. The ApoE-deficient mice fed the cholesterol and oxysterol diets tended to have greater level of cholesterol than those fed the control diet, but the difference was not significant. Although there were no significant differences in the levels of total oxysterols

Table 2. Lipid levels and α-tocopherol concentrations in serum and liver of apolipoprotein E-deficient mice fed the control, cholesterol and oxysterol diets†

(Mean values with their standards errors for five mice per dietary group)

Diet...	Control		Cholesterol		Oxysterol	
	Mean	SE	Mean	SE	Mean	SE
Serum (mmol/l)						
Cholesterol	15·3	1·35	16·9	1·63	17·5	0·26
Triacylglycerol	1·24	0·21	1·22	0·23	1·67	0·40
Phospholipid	5·08	0·65	5·23	0·55	6·28	0·92
α-Tocopherol	0·057	0·009	0·064	0·003	0·051	0·008
Liver (μmol/g tissue)						
Cholesterol	24·7	1·35	30·1*	1·37	22·6	1·30
Triacylglycerol	198	20	175	24	174	27
Phospholipid	26·6	1·55	29·3	1·55	30·3	0·93
α-Tocopherol	0·456	0·026	0·444	0·044	0·360	0·028

Mean value was significantly different from those of the control and oxysterol groups: *$P<0·05$.
† For details of diets and procedures, see Table 1 and p. 340.

among the groups, the oxysterol diet resulted in a significant ($P<0·05$) increase in cholestanetriol and 7-ketocholesterol in comparison with the control and cholesterol diets.

Table 4 shows the levels of serum and liver oxysterols in the ApoE-deficient mice fed the control, cholesterol and oxysterol diets. Dietary cholesterol resulted in no significant effect on the levels of the serum oxysterols. Dietary oxysterols, however, prominently increased the oxysterols measured, except for 27-hydroxycholesterol. Cholestanetriol was detected only in the oxysterol group. Although 7-ketocholesterol was the most abundant species in the oxysterol diet (Table 1), the most abundant oxysterol in the serum was 7β-hydroxycholesterol, followed by β- and α-epoxycholesterol. In the liver, dietary cholesterol did not result in a pronounced effect on the levels of oxysterols. Dietary oxysterols resulted in an increase in the levels of all the oxysterols, except for 27-hydroxycholesterol. 25-Hydroxycholesterol was detected in the oxysterol group. α-Epoxycholesterol was the most abundant species, followed by 7β-hydroxycholesterol and β-epoxycholesterol. Again, the level of liver 7-ketocholesterol was relatively low.

Fig. 1 shows the lesion volume in the ApoE-deficient mice fed the control, cholesterol and oxysterol diets. There were no significant differences among the groups.

Discussion

The present study showed that diet-derived oxysterols accumulated markedly in the serum and liver of ApoE-deficient mice, although they were not prominent in the aorta, except for a significant elevation of two kinds of oxysterols (cholestanetriol and 7-ketocholesterol). Furthermore, there was no significant difference in the lesion volume in the aortic valve and the content of cholesterol in the aorta among the mice fed the control, cholesterol and oxysterol diets, indicating a less pronounced effect of dietary oxysterols on the development of atherosclerosis under the present experimental conditions. This result

Table 3. Aortic sterol levels (μg/g aorta) in apolipoprotein E-deficient mice fed the control, cholesterol and oxysterol diets†

(Mean values with their standard errors for five mice per dietary group)

Diet...	Control		Cholesterol		Oxysterol	
Oxysterols	Mean	SE	Mean	SE	Mean	SE
Cholesterol ($\times 10^{-3}$)	5·90	1·19	8·92	2·34	7·91	4·44
7α-Hydroxycholesterol	1·09	0·11	1·35	0·23	1·30	0·24
7β-Hydroxycholesterol	0·93	0·21	1·37	0·38	2·49	0·64
β-Epoxycholesterol	3·57	0·65	4·20	0·95	3·77	0·81
α-Epoxycholesterol	1·73	0·35	1·87	0·43	3·31	0·71
Cholestanetriol	1·16	0·23	1·15	0·08	1·95*	0·15
7-Ketocholesterol	1·69	0·29	1·81	0·09	2·66*	0·37
25-Hydroxycholesterol	0·26	0·02	0·42	0·08	0·31	0·10
27-Hydroxycholesterol	0·49	0·07	0·57	0·13	0·41	0·06
Total oxysterols	10·91	1·77	12·37	1·73	15·28	1·69

Oxysterols, cholesterol-oxidation products 7α-hydroxycholesterol, cholest-5-en-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-en-3β,7α-diol; β-epoxycholesterol, cholestan-5β, 6β-epoxy-3β-01; α-epoxycholesterol, cholestan-5α, 6α-epoxy-3β-01; cholestanetriol, cholestan-3β, 5α, 6β-triol; 7-ketocholesterol, cholest-5-en-3β-01-7-one; 25-hydroxycholesterol, cholest-5-en-3β,25-diol; 27-hydroxycholesterol, 25-*R*-cholest-5-en-3β, 26-diol.
Mean values were significantly different from those of the control and cholesterol groups: *$P<0·05$.
† For details of diets and procedures, see Table 1 and p. 340.

Table 4. Cholesterol-oxidation products (oxysterol) levels of serum (mg/l) and liver (μg/g) in apolipoprotein E-deficient mice fed the control, cholesterol and oxysterol diets†

(Mean values with their standard errors for five mice per dietary group)

	Serum						Liver					
Diet...	Control		Cholesterol		Oxysterol		Control		Cholesterol		Oxysterol	
Oxysterol	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
7α-Hydroxycholesterol	0·15	0·01	0·12	0·01	4·01*	0·37	0·19	0·02	0·27	0·06	0·59*	0·18
7β-Hydroxycholesterol	0·11	0·03	0·10	0·01	17·63*	1·70	0·13	0·01	0·19	0·05	1·90*	0·47
β-Epoxycholesterol	0·76	0·37	0·41	0·11	9·76*	0·74	0·15	0·08	0·20	0·09	1·53*	0·32
α-Epoxycholesterol	0·70	0·40	0·29	0·10	8·59*	0·84	0·16	0·10	0·14	0·09	4·27*	0·92
Cholestanetriol	nd		nd		0·43	0·04	0·04	0·01	0·04	0·01	0·15*	0·01
7-Ketocholesterol	0·09	0·02	0·06	0·01	2·37*	0·44	0·20	0·02	0·19	0·04	0·40*	0·08
25-Hydroxycholesterol	0·05	0·01	0·05	0·01	0·44*	0·03	nd		nd		0·10	0·02
27-Hydroxycholesterol	0·31	0·06	0·28	0·05	0·37	0·08	0·20	0·05	0·33	0·12	0·27	0·08
Total oxysterols	2·18	0·50	1·31	0·15	43·60*	3·95	1·08	0·18	1·37	0·30	8·59*	1·75

7α-Hydroxycholesterol, cholest-5-en-3β,7α-diol; 7β-hydroxycholesterol, cholest-5-en-3-β,7β-diol; β-epoxycholesterol; cholestan-5β,6β-epoxy-3β-01; cholestanetriol, cholestan-3β,5α,6β-triol; 7-ketocholesterol, cholest-5-en-3β-01-7-one; 25-hydroxycholesterol, cholest-5-en-3β, 25-diol; 27-hydroxycholesterol, 25-*R*-cholest-5-en-3β,26-diol; oxysterols, cholesterol oxidation products; nd, not detected.
Mean values were significantly different from those of the control and cholesterol groups: *$P<0·05$.
† For details of diets and procedures, see Table 1 and p. 340.

was in contrast to that of Staprans *et al.* (2000) who reported that oxysterols in their diet increased the aortic lesion area in ApoE-deficient mice obtained from Jackson Laboratories, as were those in the present study. Although the exact reason for this discrepancy is not clear, their experimental conditions differed from those of our present study.

Staprans *et al.* (2000) prepared oxysterols by heating cholesterol at 100°C for 16 h. The resulting preparations were composed of 900 g unreacted cholesterol/kg and 50–100 g of oxysterols/kg of which 25–50 g were unidentified polar compounds. The major oxysterols identified by GC were 7-ketocholesterol (400–450 g/kg total oxysterols), 7β-hydroxycholesterol (150–200 g/kg total oxysterols), β-epoxycholesterol (150–200 g/kg total oxysterols), α-epoxycholesterol (100–150 g/kg total oxysterols) 7α-hydroxycholesterol (70–100 g/kg total oxysterols).

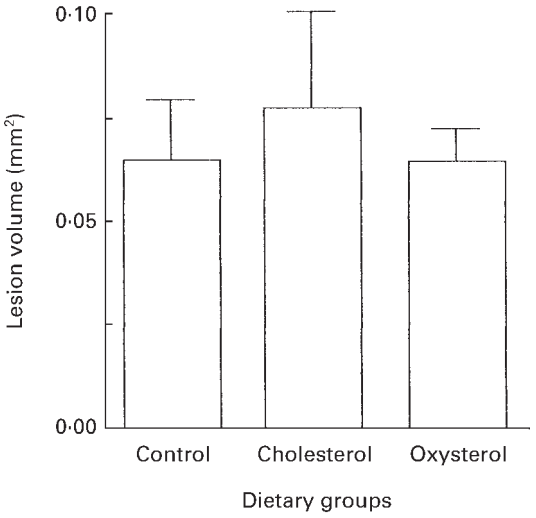

Fig. 1. Lesion volume of the aortic root in apolipoprotein E-deficient mice fed the control, cholesterol and oxysterol diets. For details of diets and procedures, see Table 1 and p. 340. Values are means for five mice per dietary group standard errors shown by vertical bars.

Therefore, the composition of the oxysterols in both experiments appeared to be similar, but not identical. The amount of dietary oxysterols might obscure the results, because Higley *et al.* (1986) reported that rabbits fed a relatively high content of oxysterols (166 mg oxyterols/kg body weight per d), had less severe atherosclerosis, in comparison with those fed a comparable amount of cholesterol. However, the amount of oxysterols in our diet (0·2 g/kg) was almost in the same range as that used by Staprans *et al.* (2000), because the final oxysterol content in their diet was between 0·07 and 0·15 g/kg. In contrast to our present diet, Staprans *et al.* (2000) used a standard mouse chow supplemented with 1·5 g cholesterol/kg (as a control diet) or 1·5 g cholesterol/kg plus an oxysterol preparation (as an oxysterol diet). Our diet contained *tert*-butyl hydroquinone, which is a recommended antioxidant in the purified diet of the American Institute of Nutrition (Reeves *et al.* 1993), and dietary *tert*-butyl hydroquinone has been reported as suppressing free radical-mediated tissue injury in streptozotocin-induced diabetic rats (Nishizono *et al.* 2000) Accordingly, dietary *tert*-butyl hydroquinone might have obscured the atherogenic effect of the dietary oxysterols by lowering the contribution of oxysterols to the overall oxidative stress. In fact, Staprans *et al.* (2000) showed increased levels of oxidized fatty acids in the serum in ApoE-deficient mice fed their oxysterol diet. Although we did not measure lipid oxidation, there was no significant reduction of α-tocopherol in the serum and liver when the ApoE-deficient mice were fed the oxysterol diet in our present study. Furthermore, dietary supplementation of antioxidants has been reported to effectively retard lesion development with ageing in the aorta and aortic valve in ApoE-deficient mice (Pratico *et al.* 1998; Terasawa *et al.* 2000; Miura *et al.* 2001) and rabbits (Freyschuss *et al.* 1993). Finally, the feeding period might obscure the results, because Staprans *et al.* (2000) fed their diet to 12-week-old ApoE-deficient mice for 4

months, whereas we fed 9–10-week-old mice our diet for 2 months. Taken together, it is likely that experimental conditions modulate the effect of dietary oxysterols on the lesion development in the arteries of ApoE-deficient mice.

In the present study, the amount of diet-derived individual oxysterol that accumulated in the serum and liver differed from the proportion in the oxysterol diet. In the diet, 7-ketocholesterol (221 g/kg total oxysterols) was the most predominant species, followed by α-epoxycholesterol (162 g/kg total oxysterols), β-epoxycholesterol (156 g/kg total oxysterols), 7β-hydroxycholesterol (152 g/kg total oxysterols), 7α-hydroxycholesterol (56 g/kg total oxysterols), 25-hydroxycholesterol (27 g/kg total oxysterols) and cholestanetriol (13 g/kg total oxysterols). In the serum, 7β-hydroxycholesterol (17·63 mg/l), was the most abundant species followed by β-epoxycholesterol (9·76 mg/l, α-epoxycholesterol (8·59 mg/l), 7α-hydroxycholesterol (4·01 mg/l) and 7-ketocholesterol (2·37 mg/l). In the liver, 7-ketocholesterol (0·4 μg/g) was the minor species and the most abundant was α-epoxycholesterol (4·3 μg/g), followed by 7β-hydroxycholesterol (1·9 μg/g), β-epoxycholesterol (1·5 μg/g) and 7α-hydroxycholesterol (0·6 μg/g). These disproportionate distributions of oxysterols in the serum and liver from that in the diet suggest that the absorption and/or metabolism of individual oxysterols differ with type. In a separate experiment, we measured the lymphatic transport of dietary oxysterols in rats (Tomoyori *et al.* 2002). The results indicated that the amount of oxysterols transported into the thoracic duct lymph was approximately proportional to the dietary content. Although an apparent recovery of the individual oxysterols in the lymph for 7 h was not proportional to the dietary content, the recovery was in the following order: α-epoxycholesterol (10·5 %), 7-ketocholesterol (5·8 %), cholestanetriol (5·2 %), 7β-hydroxycholesterol (4·8 %), 7α-hydroxycholesterol (3·4 %), β-epoxycholesterol (2·2 %) and 25-hydroxycholesterol (1·8 %). This indicates that the absorption rate of 7-ketocholesterol in ApoE-deficient mice would not be so low in comparison with the other oxysterols. It is, therefore, likely that the low content of 7-ketocholesterol in the serum and liver of ApoE-deficient mice can be attributed to the rapid metabolism in the serum and liver. A similar conclusion was reported by others (Lyons *et al.* 1999; Lyons & Brown, 2001), who found that isotopically labelled 7-ketocholesterol was rapidly cleared from the circulation and excreted into the intestine in rats. In contrast to 7-ketocholesterol, 7β-hydroxycholesterol and α-epoxycholesterol were relatively abundant oxysterols in the serum and liver of ApoE-deficient mice fed the oxysterol diet. Given their amounts in the serum and liver, dietary 7β-hydroxycholesterol and α-epoxycholesterol appeared to be slowly metabolized in the serum and liver respectively. Accordingly, we might expect a significant accumulation of these oxysterols in the aorta, but it was not the case. In the mice fed the oxysterol diet in comparison with those fed the control and cholesterol diets, it was the 7-ketocholesterol and cholestanetriol that accumulated significantly ($P<0·05$) in the aorta. Therefore, we could not predict the level of oxysterols in the extrahepatic tissues from the serum level. More studies, therefore, are required to characterize the relationship between the concentration of individual oxysterols and the deposition in the extrahepatic tissues or development of atherosclerosis.

In the present study, we could detect an appreciable amount of 25- and 27-hydroxycholesterol in the aorta of ApoE-deficient mice. The oxysterol diet, however, did not result in a significant increase in these oxysterols in the aorta. Since 27-hydroxycholesterol was not present in the diet in the present study, it must be derived by the aortic synthesis from cholesterol in response to the accumulation (Björkhem *et al.* 1994). The present results support the important role of the arterial 27-hydroxylase in removing cholesterol from the extrahepatic tissues to the liver where they are oxidized into bile acids (Björkhem *et al.* 1999).

In summary, the present results indicate that the dietary oxysterols accumulate to a greater extent in the serum and liver than in the aorta in the ApoE-deficient mice. Furthermore, dietary oxysterols do not result in a significant increase in the aortic cholesterol level or the lesion volume in the aortic valve. These results indicate that exogenous oxysterols do not promote the development of atherosclerosis in ApoE-deficient mice.

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